

Energy metabolism and performance of *Mytilus galloprovincialis* under anaerobiosis

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Intertidal individuals of *Mytilus galloprovincialis* were exposed to anaerobiosis in laboratory at 22°C and a set of biochemical metabolites and survival potential determined. Differences in survival potential between individuals emersed or kept in oxygen-free seawater were residual according to ST₅₀ values (survival time, $P \approx 0.05$) but emersed individuals survived significantly longer when considering ST_{90–100} values ($P < 0.05$). Anaerobiosis was similarly activated under both emersion and incubation in anoxic seawater after 6 h according to a seven-fold increase in succinate. Longer exposure of individuals (up to 48 h) caused succinate (and propionate) to increase but in a higher magnitude under incubation with anoxic seawater. Propionate appeared in soft tissues after 24 h of incubation in anoxic seawater and after 48 h when individuals were emersed. Glycogen was not utilized after 6 h in any case, but was progressively used with longer exposure times and in a higher magnitude under incubation in anoxic seawater (48 h). Adenylate energy charge (AEC) was highly affected by both exposure time ($P < 0.001$) and anaerobic treatment ($P < 0.01$). Rapid breakdown of ATP and phospho-L-arginine (PLA) did occur during the first 24 h of anaerobiosis, the latter ATP drop was accompanied by slight increase of ADP but strong increase of AMP that accumulated in a higher magnitude under incubation in anoxic seawater. Biochemical results of the present study suggested a certain degree of aerobiosis for emersed *M. galloprovincialis* that in turn is linked to a slight but significant longer survival performance. Most significant biochemical changes occurred during the first 24 h of oxygen deprivation, but significant differences between treatments were observed after 24–48 h. These lag differences in biochemical metabolites together with more accurate survival analyses have to be considered when investigating the energy metabolism linked to the anaerobic performance of *M. galloprovincialis*.

INTRODUCTION

The littoral zone represents a highly variable environment that significantly affects the metabolism of marine molluscs because of the incidence of cyclic exposure to air and consequently, intermittent hypoxia/anoxia that caused important changes in the energy metabolism of animals. Survival in air has been suggested as a useful tool to reflect even finer differences than other parameters such as condition factor, feeding rate and glycogen content (Thomas et al., 1999). A link between anaerobic performances as survival capacity and biochemical data has been demonstrated between *Venus gallina* and *Scapharca inaequivalvis* (Brooks et al., 1991) and for individuals of *Littorina saxatilis* (Sokolova et al., 2000). A large body of data is available on mussels' mortality in air, anaerobic end product accumulation, anaerobic depletion of glycogen, adenylates and phosphagens (Eertman et al., 1993; Isani et al., 1995; Sukhotin & Pörtner, 1999 among many others) but less attention has been focused on the anaerobic performance of individuals with regard to biochemical analyses for the specific case of *Mytilus galloprovincialis*. A certain degree of aerial respiration and the potential to perform metabolic arrest by inducing metabolic suppression (energy conservation) are the most

important strategies that individuals carry out to cope with oxygen deprivation in the littoral zone (Widdows et al., 1979; Hochachka, 1985; Shick et al., 1988; Storey et al., 1990). The capacity of aerial respiration for the specific case of *M. edulis* and *M. galloprovincialis* have been reported to represent 6–17% of aquatic rates (Widdows et al., 1979). The aim of this study was to investigate a number of biochemical changes that the mussel *Mytilus galloprovincialis* activates under environmental restrictions represented by air exposure (emersion) at 22°C and compared to lack of oxygen in the seawater. A comparison between biochemical data and the eventual performances in terms of survival will be discussed.

MATERIALS AND METHODS

Sampling of individuals

Mytilus galloprovincialis was collected at an intertidal location exposed to active wave action in the outer area of Ría de Vigo (north-western Spain) in summer. Daily average emersion time in the field was around 30% and, seawater and air temperature were 15°C (14–16°C summer average values) and 15–28°C taking into account the daily cycle of tidal exposure, respectively. Mean values of shell

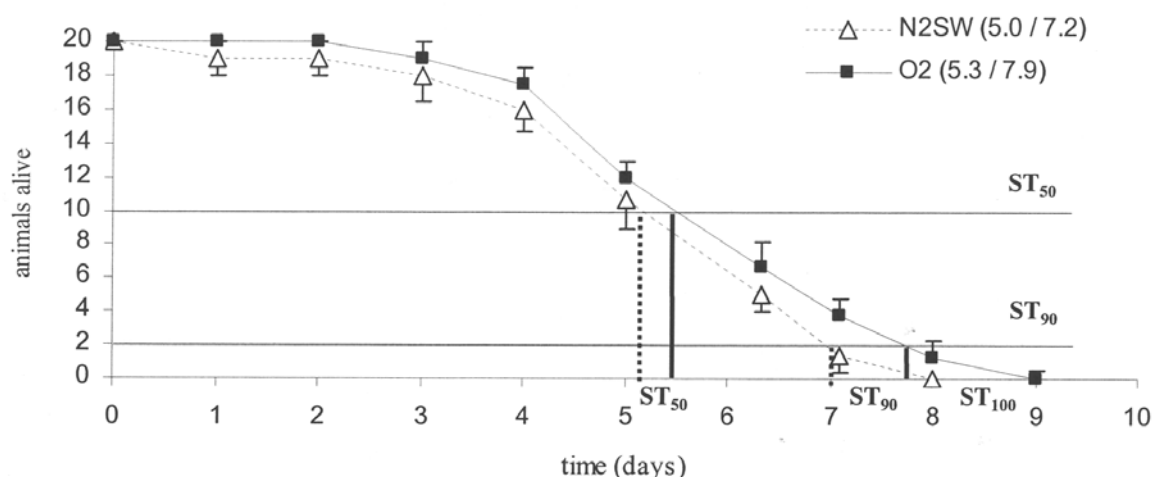


Figure 1. Survival profiles of *Mytilus galloprovincialis* individuals subjected to air exposure (O_2) and incubation in anoxic seawater (N_2SW) at 22°C. $ST_{50/90}$ values are shown in parentheses.

length and tissue dry weight were 50.4 ± 2.7 mm and 0.93 ± 0.40 g, respectively ($N=25$). Condition index values were 4.02 ± 0.83 mg of soft tissue dry weight per cm^3 . Individuals were immediately transported to the laboratory, placed in an aquarium with well-aerated running seawater and a mixture of microalgae (Tahitian *Isochrysis* aff. *galbana*, T-ISO) and sediment (40:60 microalgae:sediment, by weight) supplied with a peristaltic pump at constant flow (1.2 mg of particulate matter per litre; 50% of organic weight). Salinity and temperature were kept at 31 ppt and 22°C, respectively and individuals were maintained until the experiment was conducted at day five.

Protocol of the experiment

Two replicates of 20 individuals each were exposed to air in a chamber at 22°C on filter paper with continuous humidity for survival measurements. Anoxic seawater was prepared by vigorously bubbling filtered seawater for approximately 2 h with nitrogen gas in glass reservoirs (oxygen concentration measured lower than 0.15 mg l^{-1}). From these reservoirs water was siphoned into 2-l conical jars while flushing nitrogen gas over the surface. Twenty individuals were introduced in the jars occupying the bottom layer. The flasks were tightly closed with rubber stoppers and only opened shortly once a day to check mortality. Spilled medium as consequence of removing dead animals within the flasks was replaced by freshly prepared anoxic seawater. No food was added during anoxic incubations and incubation media (2-litre) were not exchanged (static systems). Mortality was followed daily. In parallel, a similar number of animals to those used for survival potential (replicates of 20 individuals) were similarly incubated under oxygen-free seawater and emersed on filter paper with the aim of collecting biomass for biochemical determinations after 6, 24 and 48 hours.

Tissue sampling, extraction and analysis of metabolites

Individuals were sampled and rapidly dissected by excising adductor muscle. Whole soft tissues were collected, placed on a bed of broken ice and squeezed between aluminium

blocks that had been pre-cooled in liquid nitrogen. A number of individuals ($N=3$) were homogenized as a sample and three samples each with three different individuals were considered. Control individuals were sampled from the aquarium maintained under aerobiosis at 0, 6, 24 and 48 h of the experimental time. Before preparing the extracts, frozen tissues were lyophilized for 48 h, dry tissues were then powdered, weighted and samples of approximately 100 mg (adenylates and phospho-L-arginine) and 400 mg (anaerobic metabolites) were homogenized in ice cold 7% perchloric acid (PCA) using an ultra-Turrax (4×30 s at maximum speed). The homogenate was centrifuged (30 min, 40000g) and an aliquot of the supernatant was adjusted to pH 7–8 with 5M K_2CO_3 and centrifuged again to spin down potassium perchlorate. Adenylate analyses were carried out in a HPLC system equipped with a programmable pump Beckman 126, an ultraviolet-visible detector Beckman 167, a Reodyne injector, reverse phase Kromasil 100 C18 5 μm column (15×0.4) (Teknokroma) and a software system Gold (v. 8.1). For mobile phases used, solutions, flow and detection limits, we refer to Özogul et al. (2000). Nucleotide standards (ATP, ADP and AMP) were purchased from Sigma-Aldrich Chemical Company. Adenylate energy charge (AEC) was established according to Atkinson (1968) and the formula: $EC = [(ATP + \frac{1}{2}ADP) / (ATP + ADP + AMP)]$. Phospho-L-arginine (PLA) values were determined spectrophotometrically by using enzymatic tests according to Bergmeyer (1984) and glycogen was quantified as glucose by the phenol-sulphuric acid method (Strickland & Parsons, 1968) after precipitation with 100% ethanol.

Anaerobic end products succinate and propionate accumulated in the tissues of *M. galloprovincialis* were determined by HPLC with an ION 300×7.8 mm column equipped with a pre-column IONGUARD, that permits separation of a mix of sugars, organic acids, alcohols based on its interactions with a cationic exchange column through an isocratic flow of 6 mM H_2SO_4 at 0.4 ml min^{-1} , the signals being detected by refractive index. Injection volume was 20 μl and column temperature was maintained at 65°C. The method produced linear results for the metabolites studied

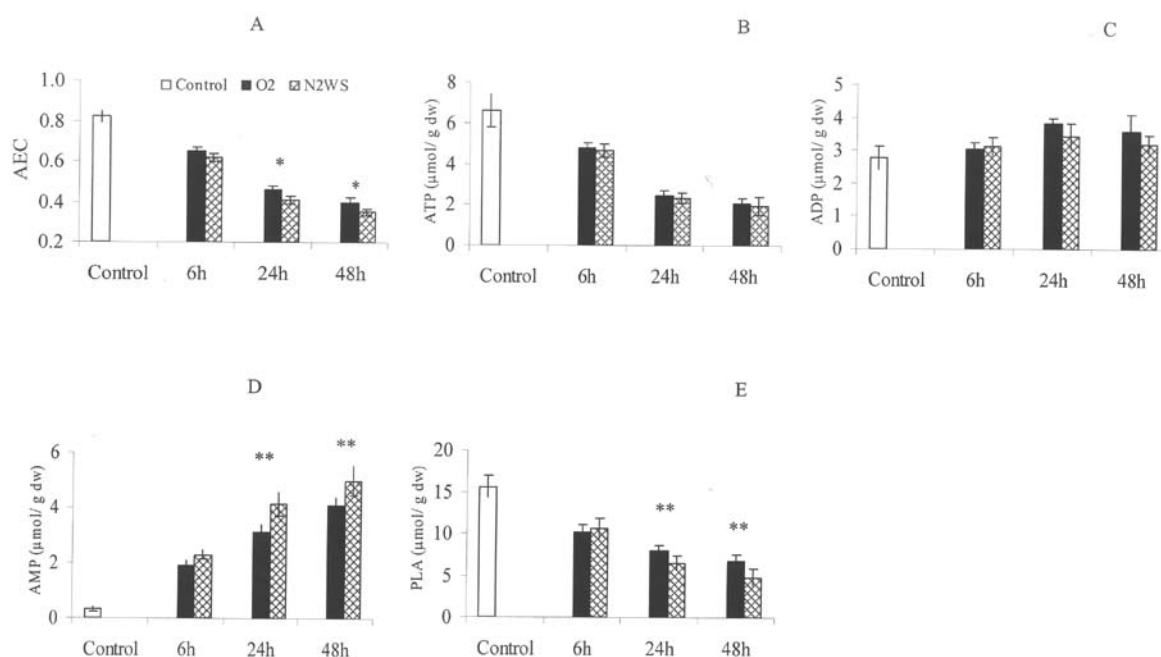


Figure 2. (A) Adenylate energy charge (AEC); (B) ATP; (C) ADP; (D) AMP; and (E) phospho-L-arginine (PLA) concentrations as μmol per gram of dry weight for the whole tissues of *Mytilus galloprovincialis* exposed to air (O_2) and incubated in anoxic seawater (N_2SW) for 6, 24 and 48 h. Control values represented the average values of those individuals kept in aerobiosis during the latter experimental times. Significant differences are shown with asterisks (**, $P < 0.01$; *, $P < 0.05$).

within the concentration range $0.3\text{--}12\text{ mmol l}^{-1}$. Constant additions methodology (4.5 mmol l^{-1}) was used for testing the linearity of the method as well as the responses obtained in different points of this linear profile. Standards were purchased from Fluka Chemie GmbH Buchs.

Statistics

The non-parametric Kaplan–Meier test was used to estimate log-rank and Wilcoxon values for comparing the survival curves (Kaplan & Meier, 1958). A confidence limit of 95% was used to test the significance of differences between groups. ST_{50} and ST_{90} values (survival times) were established using the trimmed Spearman–Kärber method ($\alpha = 10\%$) (Hamilton et al., 1997) and Weibull cumulative functions (NIST/SEMATECH e-Handbook of Statistical Methods, <http://www.itl.nist.gov/div898/handbook>). One-way analysis of variance (ANOVA), two-way ANOVA, Dunnett and t -

tests were used to detect the effect of both treatment and experimental time on biochemical components after testing normality of data. Statistica v. 6.0 was used for all these analyses.

RESULTS

Survival potential

Survival profiles of *Mytilus galloprovincialis* subjected to incubation in anoxic seawater and emersion at 22°C are shown in Figure 1. The mean survival times as ST_{50} values were 5.0 and 5.3 d for individuals incubated in anoxic seawater and emersed individuals, respectively and differences were observed to be residual ($P \approx 0.05$). However, significant differences were obtained when analysing ST_{90} (7.2 and 7.9 d, respectively; $P < 0.05$), as was the case of ST_{100} values (Figure 1).

Adenylates, energy charge (AEC) and phospho-L-arginine (PLA)

Figure 2 illustrates changes in adenylate energy charge (AEC), adenylates and phospho-L-arginine (PLA) concentrations in the whole tissues of *M. galloprovincialis*. ATP concentrations did not differ between treatments ($P > 0.05$) but were significantly affected by exposure time ($P < 0.01$). ATP concentrations strongly dropped about 27% and 63% after 6 h and 24 h, respectively from initial aerobic values of $6.51\text{ }\mu\text{mol g}^{-1}\text{ dw}$ (Figure 2B). Decrease in ATP concentrations was accompanied by a slight increase of ADP concentrations but a highly significant increase of AMP concentrations with exposure time ($P < 0.01$; Figure 2C,D). The latter AMP concentrations increase was significantly higher in animals maintained under anoxic incubation (22–34%) as compared to those emersed after 24–48 h ($P < 0.01$; Figure 2D). In order

Table 1. Two-way ANOVA on logarithmic transformed values of adenylate energy charge (AEC) for soft tissues of *Mytilus galloprovincialis* as a function of experimental time (6, 24 and 48 h) and anaerobic treatment (emersion vs incubation in anoxic seawater).

Source	SS	df	MS	F-ratio	P
Intercept	1.7294	1	1.7294	4982.23	0.000
Exposure time	0.4337	3	0.1446	416.49	0.000
Treatment	0.0062	1	0.0062	17.89	0.001
Time×treatment	0.0032	3	0.0011	3.11	0.056
Error	0.0055	16	0.0003		

$r = 0.987$; $N = 24$; $df = 16$; $F = 182.4$; $P < 0.001$.

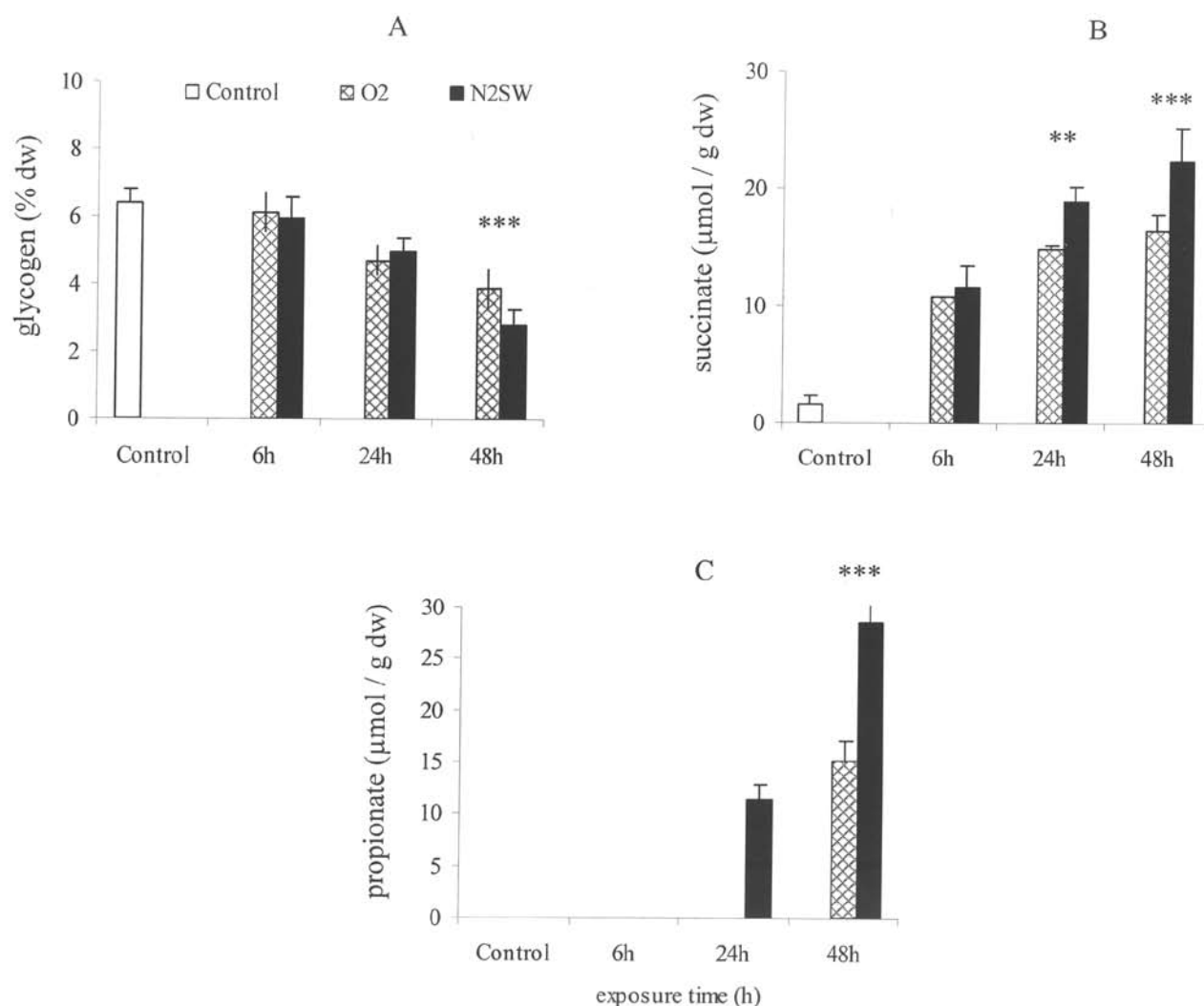


Figure 3. (A) Glycogen content as percentage values of dry weight; (B) succinate; and (C) propionate accumulation as $\mu\text{mol g}^{-1}$ dry weight for the whole tissues of *Mytilus galloprovincialis* exposed to air (O₂) and incubated in anoxic seawater (N₂SW) for 6, 24 and 48 h. Control values represented the average values of those individuals kept in aerobiosis during the latter experimental times. Significant differences are shown with asterisks (***, $P < 0.001$; **, $P < 0.01$).

to summarize the variability of total amount of adenylates with regard to both sources of variation (experimental time and treatment), two-way ANOVA was performed on integrated AEC values (Table 1). The AEC was significantly affected by both exposure time ($P < 0.001$) and treatment ($P < 0.01$; Table 1). AEC values dropped continuously with experimental time but in a higher magnitude under incubation in anoxic seawater than emersion (13%; Figure 2A). The PLA concentrations showed a significant drop of about 33% after 6 h (mean value for both treatments) (Figure 2E). Longer exposure time did cause a further drop of PLA concentrations although at a slower rate, the latter drop being more significant in individuals incubated in anoxic seawater (24–48 h) than under emersion ($P < 0.01$; Figure 2E).

Glycogen, succinate and propionate

Figure 3A–C shows the variability observed for concentrations of glycogen and anaerobic metabolites as succinate and propionate in the whole soft tissues of *M.*

galloprovincialis. Glycogen concentrations remained constant after 6 h in both treatments ($P > 0.05$) but a significant and similar drop in both treatments (24%) was observed after 24 h ($P < 0.01$). Glycogen concentrations dropped more significantly in individuals kept in anoxic seawater (53%) as compared to those emersed (39%) after 48 h ($P < 0.001$; Figure 3A). Succinate concentrations showed a similar 7-fold increase after 6 h of both treatments (emersion and incubation in anoxic seawater) ($P < 0.01$) and a 10-fold (emersed) and 13-fold (anoxic seawater) increase after 24 h ($P < 0.01$ between treatments). The latter differences in succinate accumulation were maintained after 48 h up to values of 16.4 (emersion) and 22.4 (anoxic seawater) $\mu\text{mol g}^{-1}$ dw ($P < 0.001$; Figure 3B). Propionate was not detected after 6 h of exposure but accumulated up to 11.4 $\mu\text{mol g}^{-1}$ dw after 24 h of incubation in anoxic seawater (Figure 3C). After 48 h, propionate concentrations increased by a factor of two with regard to 24 h for individuals incubated in anoxic seawater and appeared, for the first time, in emersed individuals (15.0 $\mu\text{mol g}^{-1}$ dw; Figure 3C).

DISCUSSION

Mytilus galloprovincialis is able to behave partially aerobic at rates of approximately 6–17% of aquatic aerobic values when emersed even at a high temperature of 26°C (Widdows et al., 1979). Accordingly, a risk of desiccation that could make aerobiosis inappropriate at high temperatures is not likely to occur here with *M. galloprovincialis* maintained at 22°C. The temperature tested in the present study represents an average summer value at the experimental location and biochemical data evidenced that *M. galloprovincialis* was partially aerobic under emersion (Figures 1–3). First, a lower consumption of the energy reserves as PLA and glycogen was observed in emersed individuals after 24–48 h (Figures 2E & 3A) as compared to mussels kept in anoxic seawater. Second, emersed individuals were also characterized by presenting lower concentrations of succinate and propionate as representative anaerobic end-products (Figure 3B,C) as compared to mussels kept in anoxic seawater. Since aerobiosis would support metabolism with higher efficiency in terms of ATP output (De Zwaan, 1983), better performance might be expected in terms of survival under emersion. The latter relationship was not strongly supported by differences observed in long-term survival profiles measured as ST_{50} values (5% higher values in emersed individuals; $P \approx 0.05$) but ST_{90} – ST_{100} values have to be also considered in order to draw a more complete scheme. *Mytilus* sp. is characterized by the fact that differences between survival under emersion and anoxic incubation in seawater are of less importance (unpublished results), especially when compared with another well-known gaper species, i.e. *Cerastoderma edule* (Babarro & De Zwaan, 2001) that is able to maintain aerobic rates of 28–78% of aquatic normoxic values (Widdows et al., 1979). However, *Mytilus* sp. also represents an active gaper that can behave anaerobically and aerobically simultaneously (Shick et al., 1986). Anaerobiosis was similarly activated after 6 h of emersion and incubation in anoxic seawater according to succinate values (Figure 3B), but the fact that anaerobic substrates (glycogen and PLA) and products (succinate, propionate) followed different patterns after 24–48 h highlighted a more activated metabolism under anoxia in oxygen-free seawater that might be linked to slight but significant differences in long-term survival capacity as ST_{90} and ST_{100} (Figure 1).

An activated anaerobic metabolism under anoxia was also observed for other bivalves such as *Mytilus edulis* (Zurburg & Kluytmans, 1980; Zurburg & Ebberink, 1981) and *Scapharca inaequivalvis* (Kluytmans et al., 1983), the basis for this physiological change being related to a higher activity of individuals incubated in anoxic seawater that would include valve movements exploring surrounding water (Zurburg & Ebberink, 1981) and also the possibility that oxygen contributed to some extent in the ATP production under emersion. More recently, factors usually neglected to play a significant role in experimental anaerobiosis, i.e. proliferation of anaerobic bacteria (Babarro & De Zwaan, 2001) have been reported to interfere with the anaerobic metabolism of individuals and its potential for metabolic arrest (De Zwaan et al., 2002). Indeed, such bacterial proliferation and the concomitant formation of sulphide occurred in our long-term anoxic incubations in seawater

(data not shown) and might be responsible for hypothetical differences in long-term metabolic arrest of individuals. Bacterial proliferation in static incubations of *Mytilus edulis* for nine days at 18°C using a similar experimental design to that in the present study was reported to represent values of approximately 12.10^6 bacteria ml^{-1} and after a lag period of 2–3 d (Babarro & De Zwaan, 2002).

Differences in biochemical metabolites observed in the present study for the period 24–48 h cannot be linked, however, with the deterioration of the anoxic (static) system that normally occurs later when mortality begins, but established the basis for a slight but statistically different long-term anaerobic performance of individuals (Figure 1). As a general rule, it has been reported that a lower metabolic rate is related to a higher anoxic survival capacity of individuals (Brooks et al., 1991). More detailed analysis of survival potential is recommended for establishing links between energy metabolism and anaerobic performance especially in species with both aerobic and anaerobic metabolisms operative under stress.

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